Blue light induces DNA damage in normal human skin keratinocytes

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Blue Light induces DNA-damage and may contribute to chromosome aberrations in normal human skin keratinocytes

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Abstract

Background: The generation of DNA damage by ultra-violet radiations (UV) is well established, and both the nature of the DNA lesions and their respective DNA-repair pathways, have largely been described. Besides UV rays, visible light constitutes a very important part of the sun spectrum where blue light is considered a significant contributor to premature aging. However, blue light-induced DNA damage has not been deeply explored yet.

Methods: In the present study, we assessed in human skin keratinocytes the DNA and Chromosome damaging activities of blue light rays (415 nm) as well as their associated DNA-repair mechanisms.

Results: Our results demonstrated that blue light induced dose-dependent DNA-damage in human keratinocytes. Both Oxidative and Cyclobutane-Pyrimidine-Dimers (CPD) DNA-lesions were generated. They were repaired through base excision repair (BER) and nucleotide excision repair (NER) pathways, respectively. Moreover, by using the Micronucleus assay we demonstrated, for the first time, that a blue wavelength exerted a clastogenic/aneugenic effect in human keratinocytes, leading to chromosome heritable aberration.

Conclusion: We concluded that, in normal human keratinocytes, blue light creates genotoxic lesions which might accelerate or at least contribute to premature skin aging.

Keywords

Blue light, DNA damage, DNA repair, keratinocytes

1. Introduction

Blue light (400-500nm) takes part of the visible electromagnetic spectrum. Natural sunlight blue wavelengths, which are always mixed with green, yellow and red wavelengths, have been shown to play an important role in the regulation of sleeping patterns and circadian rhythms.[1] However, since the 1980s the development of light-emitting diodes (LED) without red and near infrared wavelengths, and the concomitant exponential use of digital technologies, such as flat-screen TVs, computers, tablets and smart phones, have led to a drastic increase of human exposure to blue light [2] and to several concerns about their possible involvement in the development of degenerative eye diseases. Moreover, the expansion of therapeutic protocols using blue light irradiation in several dental treatments and dermatologic therapies, has highlighted their potential side-effects.[3]

The toxic and genotoxic effects of blue wavelengths have mainly been studied in the context of their therapeutic and ocular risks: they have been shown to induce cellular dysfunction and cell death in gingival fibroblasts [4] and lens epithelial fibroblasts [5], to generate sister-chromatid exchanges and endoreduplicated chromosomes in CHO cells [6], and to cause deoxyribonucleic acid (DNA) strand-breaks in retinal pigment epithelial cells.[7] Blue wavelengths near ultra-violet A (UVA) (400-450 nm), shown to be the most energetic visible wavelengths, have been supposed to produce the most important adverse effects in mammalian cells [8], mainly through the extracellular and intracellular photosensitized production of Reactive Oxygen Species (ROS). [9] However, direct interactions with DNA-bases have been also demonstrated. [7] Concerning cutaneous exposure, it has been shown that blue wavelengths penetrate into the skin [10] and induce both ROS production and cell death.[9, 11, 12] Recent studies have shown that blue light can also induce DNA damage in keratinocytes [13, 14] but the identification of the DNA lesions have been poorly studied.

In the present work, we attempted to study the DNA-damaging activity of blue light (415 nm), its DNA-repair mechanisms, and its resulting chromosome damage in human skin cells. Blue light induced DNA-damage and DNA-repair were evaluated by the comet assay in human normal keratinocytes, the predominant cell type in the epidermis.[15, 16] Chromosome mutations resulting from unrepaired DNA double-strand breaks and chromosome segregation abnormalities were measured by the micronucleus assay.[17]

2. Materials and methods

2.1. Chemicals and cell cultures

All reagents for cell cultures were purchased from Dutscher (Brumath, France), except bovine pituitary extract, recombinant epidermal growth factor (rEGF) and keratinocyte serum-free medium (K-SFM) which were from GIBCO-BRL (Life Technology, Cergy Pontoise, France). All chemicals for the comet and the micronucleus assays were purchased from Sigma–Aldrich (Saint Quentin Fallavier, France). Human Epidermal Keratinocytes Neonatal were purchased from ScienCell Research Laboratories. They were maintained in “Keratinocytes-SFM” medium supplemented with bovine pituitary extract (30 µg/ml) and recombinant epidermic growth factor (rEGF, 0.2 ng/ml). Cultures were performed in a 37°C humidified incubator with 5% CO_2 atmosphere.

2.2. Irradiation procedure

Blue light irradiation was carried out with a Biolambda blue light irradiator (Biolambda, Sao Paulo, Brasil) equipped with 415 nm LED, at an irradiance of 8 mW/cm². Before irradiation, well covers were removed and cells were irradiated with 4.8 J/cm², 9.6 J/cm², and 14.4 J/cm² for the standard comet assay and the micronucleus assay, and 9.6 J/cm² for DNA-repair kinetics.

UVA/visible irradiation was carried out with a Suntest CPS+ solar simulator (Atlas Material Testing Technology BV, Moussy le Neuf, France) equipped with a xenon arc lamp (1100 W) and special glass filters (ID65) restricting transmission of light below 290 nm and near IR-blocking filter. The irradiation intensity was fixed at 76.5 mW/cm², and cells were irradiated with 15 J/cm^2. UVB irradiation was carried out with an UV irradiator Bio-Sun (Vilbert Lourmat, Marne la Vallée, France). The irradiator was equipped with a monochromatic UVB lamp, which delivered a calibrated computer-assisted beam of 312 nm wavelength at an irradiance of 0.91 mW/cm^2, and cells were irradiated with 0.08 J/cm². Blue-light, UVA and UVB doses have been selected for their low impact on

cell survival, as assessed by neutral red uptake assay (more than 90% cell viability as compared to the non-irradiated cells) [18].

2.3. Comet assay

The standard alkaline comet assay was performed as described by Singh et al. [19], and a modified protocol has been used to improve the sensitivity of the assay by the use of specific enzymes: FPG, the formamidopyrimidine DNA glycosylase, which allows detecting 8-oxoguanine and other purine oxidation products formed when cell nuclei are exposed to oxidative stress and which specifically recognizes oxidized DNA-bases to convert them into strand-breaks [20]; T4 endonuclease V, the specific DNA-repair protein that recognizes cyclobutane pyrimidine dimers (CPD) and cleave them to form detectable strand-breaks, allows detecting the most abundant DNA-lesions induced by UVB [21].

Briefly, cells monolayers were irradiated before trypsinization. Cells pellets were suspended in agarose and deposited onto microscope slides, then immersed in lysis solution for 90 min at 4°C. After the lysis procedure, slides were washed with BSA enzymatic buffer and treated with T4 endonuclease V, or with FPG for 30 minutes at 37°C in a humidified chamber. Electrophoresis was then carried out for 20 minutes at 25 V and 300 mA. Finally, the slides were washed with 0.4 M Tris buffer (pH 7.5), dehydrated in 100% methanol and dried for 12 hours at room temperature. Each slide was stained with 50µl of 2 µg/ml ethidium bromide and examined at 250X Magnification using a BX53-RFL fluorescence microscope (Olympus Optical Co., Tokyo, Japan) equipped with a U-MWG2 dichroic mirror (band-pass filter, 510-550 nm; long-pass filter, 590 nm) and a UPLFLN 20x objective. Image analysis was performed using the Komet software (version 6.0 Andor Technology, Belfast, Northern Ireland). A total of 50 randomly selected cells were analyzed per slide using Fenestra Komet 6.0 image analysis software (Andor Technology, Belfast, Northern Ireland). DNA damage was expressed as the Olive Tail Moment (OTM; arbitrary units); 100 OTM values were determined for each sample, 50 from each of two separate slides/sample.

The 100 calculated OTM values by sample were distributed into 40 classes between the minimal and the maximal values: each class interval corresponded to 1.4 OTM arbitrary units. A non-linear regression analysis was performed on the OTM distribution frequencies by using a χ^2 function with TableCurve 2D software (version 5.0; Jandel Scientific Software, San Rafael, CA). The calculated degrees of freedom (n) for this function were quantitative measures of the DNA damage for a sample.[22] The n was termed χ^2 OTM and was used as the sole parameter for assessing levels of DNA damage. The significance of the differences between χ^2 OTM values of non-irradiated and irradiated cells was analysed using Student's t-test. The percentage of DNA-damage (% DNA-damage) was determined as follows:

$$\%\ DNA - damage = \left[\frac{(\chi^2\ OTM_{TX} - \chi^2\ OTM_{C0})}{(\chi^2\ OTM_{T0} - \chi^2\ OTM_{C0})}\right] \boldsymbol{x100}$$

χ^2 ***OTM$_{TX}$***: χ^2 **OTM** of irradiated cells at each incubation time
χ^2 ***OTM$_{CO}$***: χ^2 **OTM** of non-irradiated cells
χ^2 ***OTM$_{T0}$***: χ^2 **OTM** of irradiated cells at T=0

2.4. Micronucleus assay

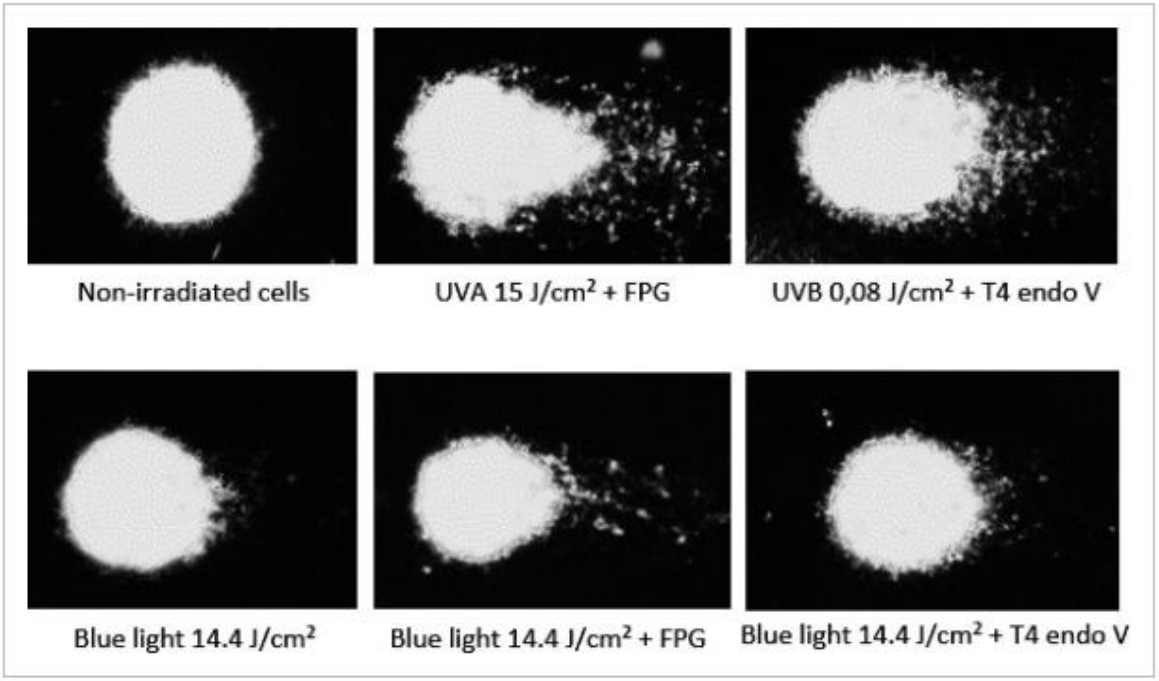
The micronucleus assay was performed according to the cytokinesis-blocked standard method.[23, 24] Keratinocytes were cultured for 24 hours in tissue culture chambers. Culture medium was replaced by sterile PBS and cells were submitted to irradiation. After irradiation, cells were grown in complete culture medium containing 3 µg/ml of cytochalasin B for 48-hour. Cells were fixed with methanol and stained with 10% Giemsa for 30 minutes. Micronuclei analysis was performed under a microscope at x1000 magnification. The rates of micronuclei were evaluated for the presence of independent nuclear core entities in a total amount of 5,000 binucleated cells. Micronuclei were identified as small nuclei well differentiated from cell nucleus, stained in the same manner and having a diameter less than one third of that of the cell nucleus. Micronuclei rates obtained for different exposure conditions were compared to the negative control by a Chi-squared test of independence in contingency tables.

3. Results

3.1. Blue-light dose-dependent DNA-damage

Figure 1 displays examples of intact and damaged nuclei observed with fluorescent microscope for the comet assay. Figure 2 displays Olive Tail Moment (OTM) distributions and the corresponding χ^2 OTM in human keratinocytes irradiated with blue light at different irradiation times. Nuclei appeared as condensed spherical spots with low levels of single-strand breaks in non-irradiated cells (OTM median = 0.32 - χ^2 OTM = 2.06 ± 0.03). In irradiated cells, nuclei appeared as the shape of a comet, with a small tail formed by the migration of DNA fragments from the nucleus towards the anode. Blue light-irradiation generated a dose-dependent increase of DNA-damage: a significant photo-induced DNA-damage was obtained from an irradiation dose of 9.6 J/cm² (OTM median = 2.21 - χ^2 OTM = 2.43 ± 0.26, P<0.001).

FIGURE 1. Examples of intact and damaged nuclei observed with fluorescent microscope for the comet assay



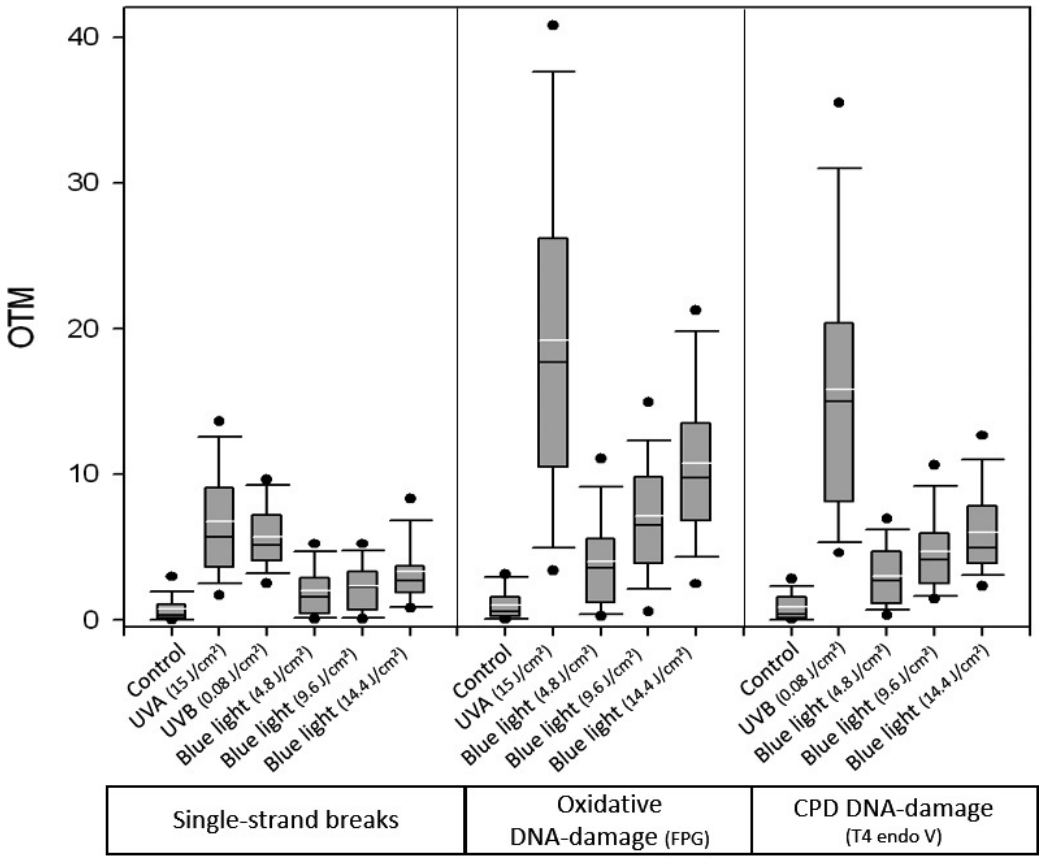
3.2. Blue-light oxidative DNA-damage and CPD-lesions

In blue-light irradiated cells, a significant dose-dependent increase of tail moment (OTM and χ^2 OTM) was observed from the lowest exposure dose of 4.8 J/cm² (Figure 2), with FPG (median = 3.55 - χ^2 OTM = 2.99 ± 0.11) or T4 endo V (median = 2.66 - χ^2 OTM = 2.94 ± 0.12). Cleavage of oxidized bases and base-dimers by FPG and T4 endo V respectively resulted in a strong enhancement of the tail moment, indicating that blue light generated both oxidative DNA-damage and CPD-lesions.

3.3. DNA-repair kinetic of oxidative DNA-damage and CPD-lesions

An immediate time-dependent decrease of DNA-lesions could be observed in all the irradiated cells, indicating the rapid involvement of DNA-repair mechanisms after irradiation (Figure 3). Oxidative DNA-lesions, detected through the cleavage of oxidized-bases into apurinic sites by FPG, were totally repaired after a 60-minute incubation period for UVA/visible light irradiation, and after a 40-minute incubation period for blue light irradiation. The repair of CPD-lesions, identified through the cleavage of glycosyl and phosphodiester bonds by T4 Endo V, was slower. A consistent decrease of CPD-lesions could be observed during the first 6 hours after irradiation. Then DNA-repair was less efficient and, after 24 hours, the percentage of unrepaired CPD reached 13% in blue light exposed cells and 40% in UVB-exposed cells.

FIGURE 2. OTM and χ^2 OTM distributions in human keratinocytes exposed to UVA, UVB, and blue light (Comparisons between non-irradiated cells and irradiated cells were performed using Student's *t* test. ***: $P < .001$)



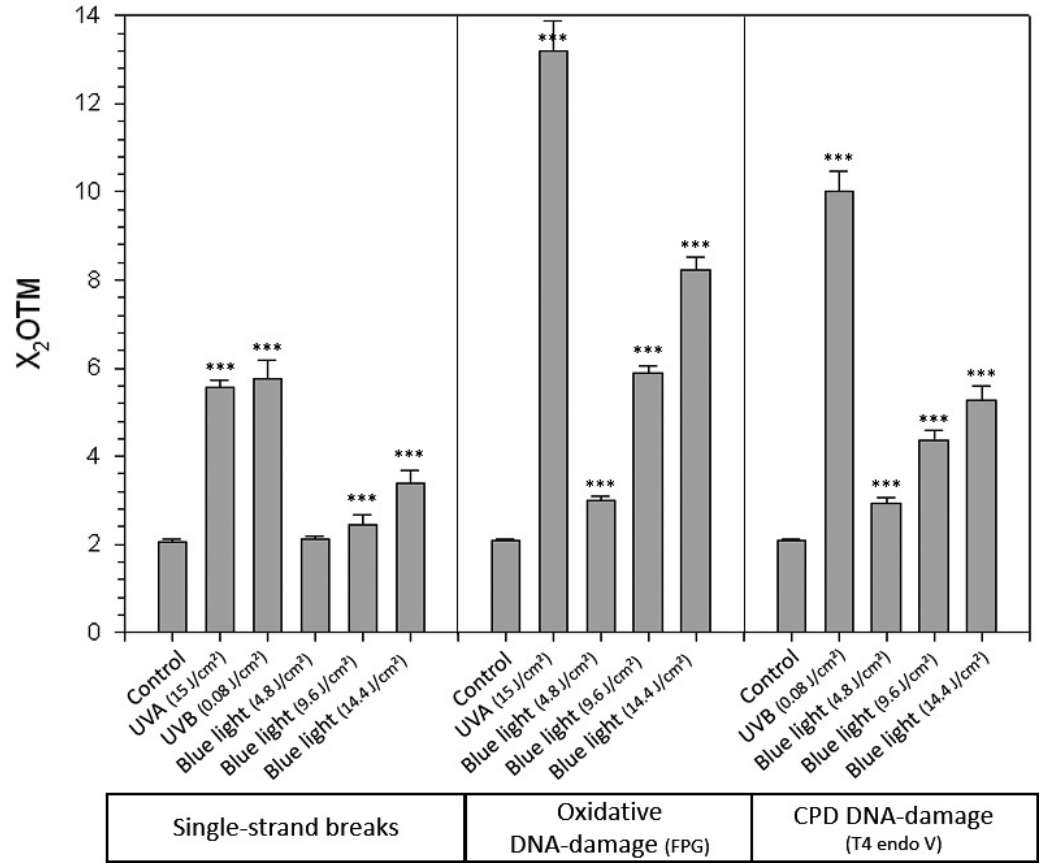
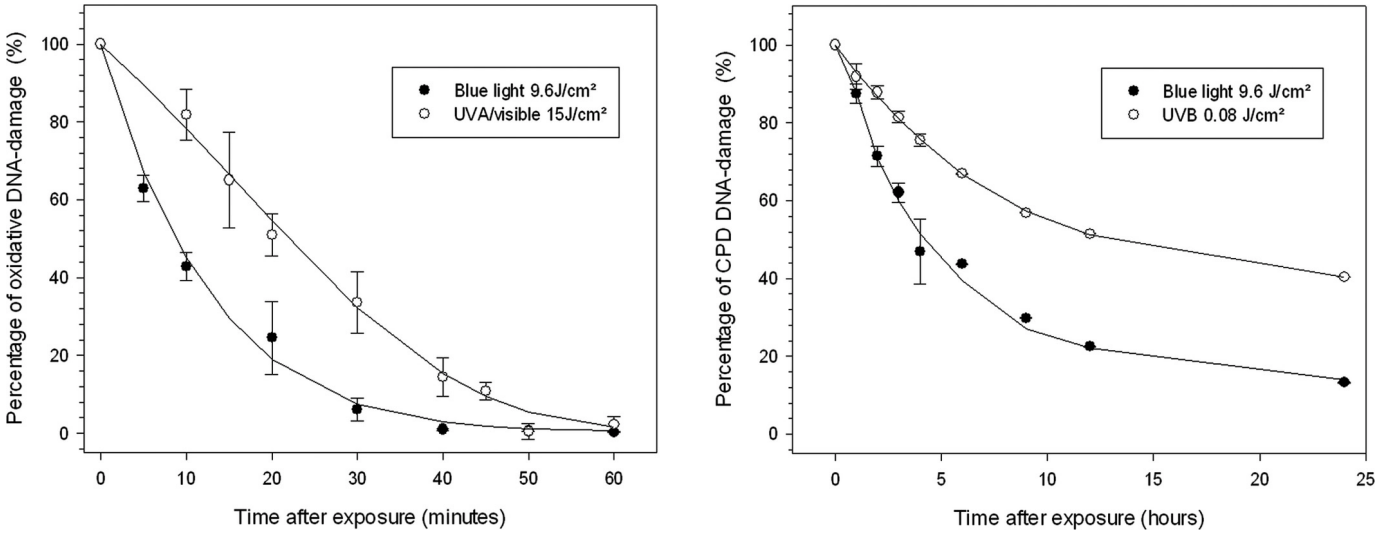


FIGURE 3. DNA repair kinetics on keratinocytes exposed to UVA/visible, UVB, and blue light (mean ± SD, n = 4 independent experiments)



3.4. Mutagenic impact of blue light irradiation

Micronuclei appeared as small nuclear inclusions in the cytoplasm of interphase cells (Figure 4), with diameters varying between 1% and 9% of the cell diameter. In blue light irradiated cells, a dose-dependent increase of micronucleated cell levels could be observed (Table I). Induction of micronucleated cell was not significant for the irradiation doses of 4.8 and 9.6 J/cm² (mean = 9.80 ± 0.84‰ - median = 9.90‰ and mean = 12.60 ± 2.07‰ - median = 12.00‰ respectively), but it became significant for the irradiation dose of 14.4 J/cm² (mean = 18.60 ± 2.07‰ - median = 19‰), suggesting that some blue light induced DNA-lesions turned into unrepaired double-strand breaks, leading to stable and heritable chromosomal damages.

FIGURE 4. Examples of binucleated keratinocytes with micronuclei

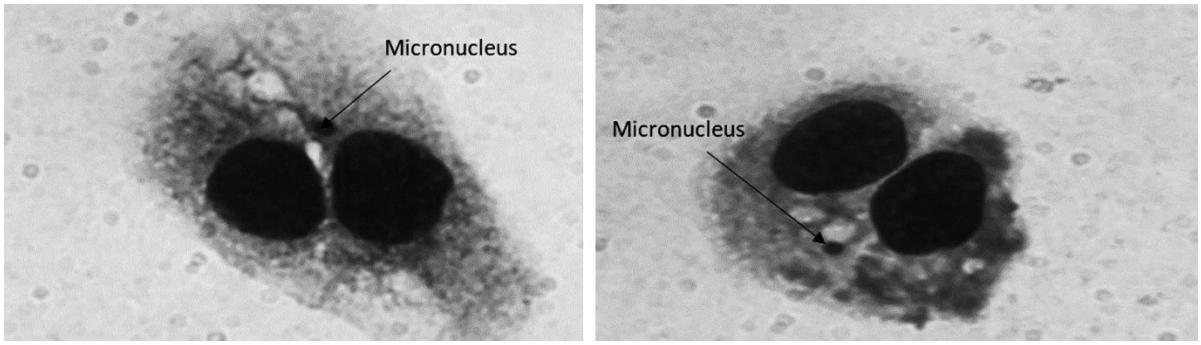


TABLE 1. Mutagenic impact of UVA/visible, UVB, and blue light in human keratinocytes by the micronucleus assay

Samples		Micronucleated cell rates ‰							
		MNC-1	MNC-2	MNC-3	MNC-4	MNC-5	*Mean ± SD*	*Median*	*P*
Non-irradiated cells		11	8	10	10	5	8.80 ± 2.39	9.40	
UVA 0.08 J/cm²		34	30	29	33	31	31.40 ± 2.07	31.00	***
UVB 15 J/cm²		20	19	14	15	16	16.80± 2.59	16.00	***
Blue-light irradiated cells	4.8 J/cm²	10	11	9	10	9	9.80 ± 0.84	9.90	
	9.6 J/cm²	12	13	12	16	11	12.60 ± 2.07	12.00	
	14.4 J/cm²	20	17	16	19	21	18.60 ± 2.07	19.00	***

MNC-1, ..., MNC-5: Micronucleated cell rates observed in 1,000 binucleated cells (‰)

P: Probability of the difference between micronucleated cell rates of non-irradiated cells and the other cells analyzed by the Chi-squared test (***: P <0.001)

4. Discussion

In the present study, the alkaline comet assay and the micronucleus assay were used to characterize the genotoxic impact of blue light in skin cells and to evaluate the cellular response to blue light induced DNA-damage. Irradiation by 415 nm wavelength, from the exposure dose of 9,6 J/cm², clearly induced dose-dependent DNA-damage in human keratinocytes and confirmed previously published data observed in both skin and retinal epithelial cells.[7, 14]

DNA-lesions were significantly enhanced in the presence of FPG, suggesting that blue light induced oxidative DNA-damage. This data was consistent with previously published studies which established that blue wavelengths could interact with intracellular porphyrins and flavoproteins to generate reactive oxygen species (ROS) [9, 25, 26] such as superoxide, nitric oxide and peroxynitrite.[27] In UVA-irradiated cells, the indirect formation of oxidized DNA bases through excited chromophores is considered the main genotoxic mechanism leading to the formation of 8-oxo-guanine and in a lower extend, DNA strand breaks and oxidized pyrimidines.[28, 29] We could suppose that blue-light may induce oxidative DNA-damage according to the same mechanisms as UVA, through an indirect oxidative process.

Surprisingly, DNA-lesions were significantly increased in the presence of T4 endo V, suggesting that blue light could also induce CPD-lesions. CPD-lesions are mainly due to high-energy photons, which are directly absorbed by DNA bases, and allow the formation of covalent bonds between two adjacent pyrimidines.[30, 31] They have been shown the predominant DNA-damage induced by UVB. Since UVA are not directly absorbed by the pyrimidine bases of DNA, the formation of CPD has long been considered negligible during UVA exposure. However, various recent studies have established that the majority of lesions induced on DNA by UVA are T-T type CPD.[30, 32] These lesions would derive from a Type I photosensitization mechanism, due to an energy transfer from a cell chromophore to the DNA rather than a direct excitation, as it is the case for UVB-induced CPD. [33] The CPD lesions observed in the present study suggested that, similarly to UVA, blue wavelengths may oxidize cellular targets to induce T-T dimers.

Two DNA-repair kinetics were obtained in human keratinocytes irradiated with blue light. In FPG-treated nuclei, a rapid decrease of oxidative DNA-damage could be observed within the first 40 minutes post-irradiation. This DNA-repair kinetic was consistent with previously published data concerning UVA-induced oxidative DNA-damage: oxidized bases are rapidly eliminated by the Base Excision Repair (BER) and, after one hour, almost all the DNA-lesions are repaired.[34] In T4 Endo V-treated nuclei, the decrease of blue light induced DNA-damage was slower, and 18% of the CPD-lesions remained unrepaired after 24 hours. This DNA-repair kinetic rate was consistent with previously published data observed in UVB-irradiated cells, suggesting the involvement of the Nucleotide Excision Repair (NER), intended to remove a wide range of DNA distorting lesions, such as CPD and 6-4 photoproducts, through a mechanism more complex than BER.

Results observed with the micronucleus assay demonstrated for the first time that a blue light could exert a clastogenic/aneugenic effect in human keratinocytes, leading to heritable chromosome aberrations.

Micronuclei are defined as chromosome fragments or whole chromosomes, which have been lost during cell mitosis, and form individual nuclear particles in the cytoplasm of daughter cells.[23] In the present study, it was not possible to selectively differentiate whole chromosomes and chromosome fragments since no FISH-technique was applied. However, the diameter of micronuclei has been proposed as an indicator of a clastogenic or aneugenic activity, acentric chromosome fragments being characterized by diameters lower than 25% of cell diameter.[35] In cells exposed to genotoxic compounds or radiations, it has been shown that a strong proportion of micronuclei arises from clastogenic events, through unrepaired double-strand DNA-breaks.[17] Our results confirmed this hypothesis, since almost all the micronuclei diameters observed in UVA, UVB and blue light irradiated cells were lower than 10% of the cell diameter. A great variety of mechanisms may transform the different DNA-lesions into acentric chromosome fragments, particularly when DNA-damage exceeds the DNA-repair capacities.[17] Oxidative DNA-lesions, such as 8-oxodeoxyguanosine, when they are in proximity or on opposite DNA-strands, may generate double-strand breaks through simultaneous excision-repair mechanisms of the BER repair pathway. Similarly, simultaneous excision events, related to the NER repair pathway, involved in the removal of DNA-lesions such as pyrimidine dimers, may also produce double strand-breaks. The presence of micronuclei in binucleated human keratinocytes may be considered a severe DNA damage since genes present in the extranuclear DNA-fragments are under-expressed or over-expressed, resulting in important phenotypic

modifications. In survival cells, chromosome abnormalities are probably transmitted from parent cells to daughter cells, leading to chromosome rearrangement and instability. [36]

The cellular impact of blue light is being increasingly investigated. But conversely, with well documented damage described on the cornea, the consequences of blue light exposure to human skin cell DNA are less predictable and have not been deeply explored yet. Results observed in the present study suggested that a single 415 nm exposure generates both oxidized DNA-bases and CPD that may turn into heritable chromosome aberrations. Moreover, human exposure to visible light, and thus to blue light, during the daily activities of life is permanent (outside, inside, behind a window), while exposure to UVA is limited to outdoor activities during the daylight. Low-dose cumulative exposures to blue light could thus induce deleterious effects that contribute to premature skin-ageing. Further *in vitro* and *in vivo* studies are necessary to investigate the genotoxic potential of blue-light and to evaluate its impact in pre-cancer skin-lesions. As interindividual variations have been shown to play an important role in human carcinogenesis through variations in intracellular oxidative/antioxidative balance or in DNA-repair pathways, experiments should be anticipated in extended cell or skin samples.[37] In addition, experiments should be proposed to evaluate the cumulative genotoxic effects of low dose repeated blue-light irradiations to better mimic the long-term human exposure.

5. Conflict of interest

None declared.

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